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13. ABSTRACT ***(Maximum 200 Words)***

We have proposed to characterize at a molecular level the recognition determinants of stimulatory and inhibitory NK receptors and the class I molecules they bind. We have pursued this via the heterologous expression of wild type and mutant NK receptors for the purpose of the determination of the atomic structure of an NK receptor/ class I MHC complex via X-ray crystallography. We have investigated several methods for the overexpression of the murine inhibitory NK receptor Ly49c. The results of this survey indicate that the most tractable solution is the over-expression of Ly49c in *E. coli*, where the protein is found in an insoluble inclusion body fraction. We have developed a protocol for the *in vitro* refolding of denatured Ly49c from this inclusion body material. This work now allows us to investigate, by in vitro killing assay and/or direct biochemical assays, the nature of the interaction between Ly49c and its ligand, H2-K^b. We have also begun screening for crystallization conditions with refolded Ly49c, for the purpose of determining its atomic structure.

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Charles A Haeseemann, J 10-19-99

PI - Signature Date

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Introduction

Natural killer (NK) cells are known to kill tumor cells, virally infected cells, and some bone marrow grafts in a pattern that is dependent on the MHC genotype of the target cell. The leading hypothesis for this selective killing of tumor cells is called the missing-self hypothesis. Natural killer cells have (at least) two types of receptors that interact with target cells. There are several versions of stimulatory receptors that interact with ubiquitously expressed molecules on target cells, including some which bind MHC class I. Ligation of these activating receptors initiates the cytolytic activity of the NK cell towards the target. A second class of receptors is inhibitory receptors. These inhibitory receptors bind the class I MHC antigens of the target cell. A successful ligation of an inhibitory receptor with a class I molecule is both sufficient and necessary to circumvent the cytolytic cascade that was initiated by the ligation of stimulatory receptors. Thus the missing-self hypothesis proposes that the targets of NK cell lysis are those cells that fail to express "normal" class I MHC antigens, and thus fail to deliver a negative signal that would otherwise rescue them from lysis. But because some activating receptors also recognize class I antigens, it is not yet clear what will be the net signal that is (not) delivered to an NK cell by a target cell which lacks class I expression. While there is little doubt that the modulation of expression of class I molecules on a tumor cell provides a growth advantage, the mechanism by which that advantage is achieved remains unknown. Because of the clear evidence that NK cells utilize the class I molecule as a trigger for both activating and inhibiting signals, and that NK cells kill tumor cells *in vivo*, it is imperative that we establish the molecular basis for these interactions. To that end, we have proposed to characterize at a molecular level the recognition determinants of stimulatory and inhibitory NK receptors and the class I molecules. We have pursued this via the heterologous expression of wild type and mutant NK receptors for the purpose of the determination of the atomic structure of an NK receptor/ class I MHC complex via X-ray crystallography.

Body

The results of our work in Months 1-12 of this study are outlined below, in the context of the approved Statement of Work (shown in bold).

Task I. Refine the expression of recombinant NKr^S in E. Coli and/or a Baculovirus expression system. Begin expression/purification of recombinant MHC class I. Priority will be given to the CD94/NKG2-A and Ly49c proteins. (Months 1-12).

- Refine conditions for refolding E.coli derived NKr (Months 1-6)

As stated in the proposal for funding, Ly49c produced in *E. coli* is always found in an inclusion body fraction, regardless of the promoter construct, induction time/temperature, or *E. coli* strain used. Thus, an in vitro refolding strategy was proposed. The vast majority of our effort in the first 12 months of this project has been directed toward developing a reliable high-yield refolding protocol. Two basic constructs were analyzed for protein production (figure 1). Both constructs include an N-terminal His_6 tag to simplify purification, and are driven by an inducible promoter. Bacteria were grown to log phase, induced with IPTG, and grown for an additional four hours.

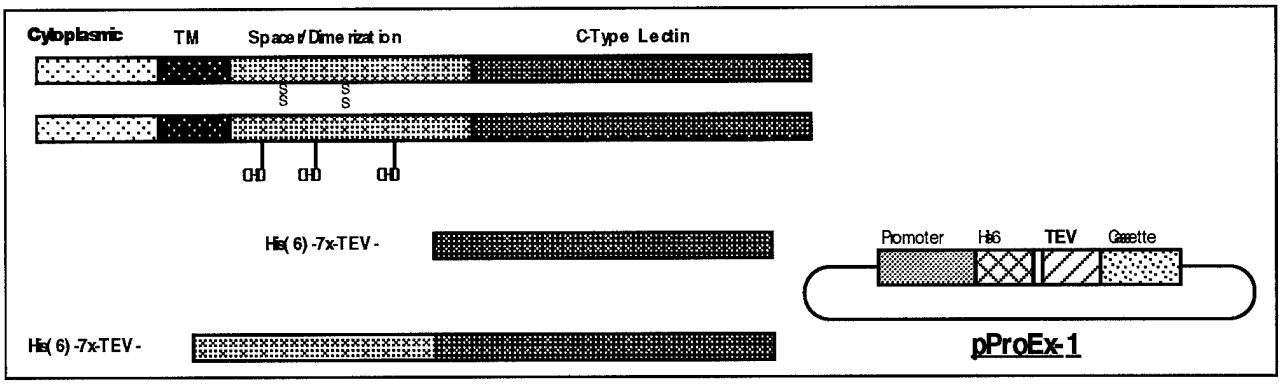

Figure 1 – Expression constructs used to generate inclusion body proteins. A schematic of a dimeric Ly49c molecule is shown at the top of the figure, with the C-type lectin, dimerization, transmembrane, and cytoplasmic domains indicated with different shading. The two basic expression constructs are shown below, being either a single C-type lectin domain with His_6 tag and TEV protease cleavable linker, or a two-domain molecule that includes the tag, dimerization, and C-type lectin domains. To the right, a basic schematic of the pProEx-1 expression vector is also shown.

The refolding experiment can be divided into three logical phases: 1) cell lysis and inclusion body isolation, 2) denaturation and purification of denatured protein, and 3) refolding, concentration, and purification of the folded protein. The following is a summary of the parameters surveyed in these three stages, and their effect on refolding.

1. Cell growth, lysis, and inclusion body isolation.

- Growth in various media (LB, M9, TB, etc.) and for different times and temperatures
  - We observed a variable amount of contaminant proteins (including significant protease activity), and found that rapid growth (37°C) in a modestly rich medium (LB) proved to yield the best ratio of recombinant protein to contaminants.

- Direct lysis of cells in Guanidine, without isolation of inclusion bodies.
  - Several literature reports claimed that the inclusion of the His_6 tag and subsequent purification by Ni^{2+} chromatography obviated the need for purification of inclusion bodies. We found that the yield and purity of our proteins was diminished by this protocol, so we abandoned it.
- Sonication of cells vs. freeze-thaw vs. extrusion vs. french press.
  - The most efficient lysis of cells (and hence, best yield of inclusion bodies) was achieved with a high-pressure extruder, with the french press a close second.
- Washing protocols to clean up inclusion bodies.
  - Including washes in various concentrations of detergents and/or urea.
- Sucrose gradient purification of inclusion bodies.
  - While the banding of inclusion bodies on a sucrose gradient proved to be a method for preparing very clean inclusion body fractions, the time and effort involved was not worth the gain in purity. The subsequent Ni^{2+} chromatography step achieved a similar level of purity.
- Mutagenesis to remove "extra" disulfide.
  - Computer modeling of Ly49c based on the structures of mannose-binding protein and E-selectin indicated that there is an additional disulfide bond in the Ly49 structures that is not present in either of these others. This disulfide bond is predicted to be in very close physical proximity to another disulfide, and could be a source of misfolding reactions based on the scrambling of these disulfides. Based on this hypothesis, we removed both cysteines of this "extra" disulfide, replacing them with serine. Subsequent expression and refolding experiments demonstrated a 5-fold increase in yield using these cysteine-mutant proteins compared to their wild-type counterparts. Thus, all subsequent experiments utilized these cysteine-free mutants.

2. Denaturation and purification of denatured protein.

- Denaturation in Urea vs. guanidine.
  - Guanidine routinely more efficient.
- Various concentrations of denaturant.
  - Increased yields with increased concentrations of denaturant, with an optimal yield at pH 6.5.
- Ni^{2+}-agarose affinity chromatography of denatured inclusion body protein.
  - Requisite step to go from 85 to 99% purity.
- Second pass Ni^{2+} affinity after concentration.
  - Significant clean-up
- $(NH_4)_2SO_4$ fractionation of protein after Ni^{2+} chromatography.
  - Achieved some fractionation, but no great gains in purity.
- Purification by DEAE ion exchange chromatography after refolding.
  - Ly49c in flow through, most contaminants stuck, so a successful purification.
- Purification by CM-cellulose ion exchange chromatography after refolding.
  - No purification achieved

3. Refolding, concentration, and purification.

- Many variations on the make-up of refolding buffers, including;

- Various protease inhibitors.
  - No effect on folding, but requisite for stability/resistance to contaminating proteases.
- Inclusion of a redox system (reduced and oxidized glutathione, at several ratios).
  - Essential
- Arginine.
  - Increased yield in presence of Arginine.
- Salts (NaCl, KCl, MgCl, CaCl, $NaPO_4$, KPO_4) in ranges from low mM to molar.
  - Concentrations greater than 100mM requisite for folding.
- Detergents (LDAO, β-Octyl glucoside, Triton X-100) at several concentrations.
  - Variable results, with marginal effect, so left out for simplicity
- pH varied over the range 4 to 9.
  - Below 8.0 and below led to precipitation during refolding.

♦ Various strategies for the final concentration of both the protein and denaturant in the final refolding mixture.
- Lower urea in refolding led to reduced yields during concentration of refolded protein.
- Any protein concentration > 10μM during refolding led to precipitation

♦ Stepped dilution versus dropwise dilution into refolding buffer.
- When stepped, the change from 2M to 1M led to precipitation, while dropwise refolding did not.

♦ *In situ* refolding on the Ni^{2+} column.
- Disastrous

♦ *In situ* refolding on a sepharose S-300 size exclusion column.
- Disastrous/proteolyzed

♦ Purification by size exclusion chromatography after refolding.
-

♦ Purification by Mono-Q ion exchange chromatography after refolding.
- Successful, but led to aggregation after concentration.

- Engineer Baculovirus transfer vectors for increased yield (Months 1-2)

Despite several new constructions of Ly49c in various Baculovirus transfer vectors, the expression was not increased to a level satisfactory for X-ray crystallography. This included engineering vectors with His_6 or His_9 tags at either the N- or C-terminus of the protein, and insertion of these constructs in several different promoter environments (i.e., canonical start codon utilization, modified Kozak consensus, polyhedron fusion). None of these various constructs yielded a significant amount of protein.

- Refine conditions for optimal Baculovirus expression (Months 3-6)

This was not attempted, since the reengineering efforts did not provide promising increases in yield.

- Develop large scale (10-100 milligram) NKr production and purification (Months 6-12)

The result of our experiments in Ly49c refolding is a protocol for the large-scale production of refolded, soluble material. At each stage of purification, protein recoveries are tracked by OD280 and/or Bradford assay calibrated by BSA. The basic protocol is as follows;

- Grow 2 liters E. coli BL21α harboring the expression vector to an OD_{600} of 0.6, shaking at 300 rpm, 37°C.
- Induce protein expression with 600μM IPTG, grow for an additional 3 hours.
- Lyse cells by homogenization in an extruder apparatus, chilled to 4°C.
- Pellet and collect the insoluble fraction by centrifugation at 6,000 rpm in a GSA rotor.
- Resuspend cell pellet in inclusion body wash buffer (6M urea, 50mM Tris•HCl pH 8.0, 50mM DTT, 50mM EDTA, 8% Sucrose, 5% Triton X-100).
- Pellet and collect the insoluble fraction by centrifugation at 6,000 rpm in a GSA rotor.
- Repeat wash in inclusion body wash buffer, pellet and collect the insoluble fraction by centrifugation.
- Solubilize the inclusion body protein in denaturation buffer A (6M Guanidine HCl, 0.5M NaCl, 10mM 2-ME, pH6.8)
- Clarify by centrifugation at 10,000 rpm in SS34 rotor.
- Apply cleared, denatured inclusion body protein to a Ni^{2+}-Agarose affinity column (1ml resin/500ml culture, packed in a Pharmacia C-column) at a flow rate of 1ml/min.
- Wash column with additional denaturation buffer A until OD280 returns to baseline.
- Elute bound protein with denaturation buffer B (same as buffer A, but pH 4.0).
- Pool eluted protein fractions and adjust to pH 8.0 with NaOH.
- Bind protein to DEAE Sepharose column in 6M Guanidine HCl, 50mM NaCl, 10mM 2-ME, pH6.8, and elute with linear NaCl gradient (50mM to 1M NaCl).
- Pool protein fractions, and apply to a second Ni^{2+}-Agarose affinity column.
- Wash with low imidazole buffer (6M Guanidine HCl, 0.5M NaCl, 10mM 2-ME, pH6.8, 40mM imidazole).
- Elute with high imidazole buffer (6M Guanidine HCl, 0.5M NaCl, 10mM 2-ME, pH6.8, 250mM imidazole).
- Refold protein by dropwise addition into stirred, 4°C refolding buffer (50mM Bis-Tris Propane, pH6.5, 150mM NaCl, Protease inhibitor cocktail [20mM PMSF, 1μg/ml leupeptin, 0.1μM bestatin, 1mM pepstatin A, 60 μg/ml chymostatin], 5mM reduced glutathione, 0.5mM oxidized glutathione, 400mM arginine). Add slowly, over a period of 2- 4 hours.
- Target concentrations for the refolding reaction are no more than 200mM urea or 10μM protein.
- After an hour of refolding at 4°C, concentrate the refolded protein (by stirred cell concentrator) to 10-20 mg/ml.

This refolded protein is stable at 4°C for 6 weeks or more, indicating that it is in fact a well-folded monodisperse protein in solution. It elutes as a symmetrical peak from size exclusion chromatography at the appropriate molecular weight. Yields from a two-liter prep are typically 15-30 mg, quite adequate for crystallography and functional experiments.

- Establish MHC class I expression/purification (Months 6-9)

Because the Ly49c refolding has taken so long to establish, we have not yet attempted large-scale production of H2-K^b. We have demonstrated at a small scale (2 liters of Schnieder cells) that we can reproduce the expression of soluble H2-K^b as reported by the Pederson group.

Task II. Develop novel high sensitivity NKr - MHC binding assays (Months 8-12).

- NK killing assay development (Months 8-10)

Preliminary attempts at an NK killing assay utilizing refolded Ly49c are just underway. We are doing this as a collaboration with the laboratory of Dr. Vinay Kumar, where a graduate student (Gene Devora) routinely assays Ly49c mediated inhibition of killing.

- ELISA/RIA development (Months 9-11)

Not yet attempted

- BiaCore Assay development (Months 10-12)

Not yet attempted

Task III. Map NKr / MHC interface via mutagenesis (Months 18-24).

Not yet attempted

Task IV. Crystallize and solve by x-ray diffraction the structure of the NKr and/or the NKr/MHC complex (Months 12-36).

- Screen conditions for crystallization (Months 12 - 24)

We have gone through two rounds of crystallization screening with refolded Ly49c. These two screens differed in the amount of urea (0 or 0.25 M) remaining in the protein solution after refolding. Neither screen resulted in any diffraction quality crystals, and further screening was deferred until killing assays (see Task II) are established to validate the activity of the molecules as bona fide active inhibitory receptors.

- Collect native data and attempt Molecular replacement (Months 15 - 24)

Not applicable at this time

- If necessary, collect heavy atom derivative data (Months 24-30)

Not applicable at this time

- Build model and refine structure (Months 24-36)

Not applicable at this time

Key Research Accomplishments

- Produced mutant Ly49c molecules, removing a disulfide bond that prevented refolding *in vitro*.
- Established a reliable protocol for the *in vitro* folding of denatured Ly49c.
- Developed a protocol for the purification and concentration of refolded Ly49c.
- Performed crystallization screens on this refolded material

Reportable Outcomes

None

Conclusions

Thus far, our research on the physical basis of NKr/MHC interaction has been mired in the technical details of *in vitro* refolding Ly49c protein from denatured inclusion body protein. We have developed a satisfactory protocol for this refolding, with a high yield of protein. We will now develop assays for the functional nature of this protein, in both an in vitro killing assay, and more direct assays of its ability to bind recombinant H-2 K^b.